Aeromicrobium marinum sp. nov., an abundant pelagic bacterium isolated from the German Wadden Sea

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An obligately salt-dependent Gram-positive bacterium, designated strain T2^T, was isolated from surface waters of the German Wadden Sea. The organism exhibited optimum growth at salt concentrations similar to that of sea water. On the basis of phenotypic, chemotaxonomic and phylogenetic differences, it is concluded that strain T2^T (=DSM 15272^T=LMG 21768^T) is the first marine species of the genus *Aeromicrobium* to be identified, for which the name *Aeromicrobium marinum* is proposed. It is also the first described marine bacterium within the family *Nocardioideae*. Strain T2^T is a rod-shaped, aerobic, heterotrophic bacterium containing LL-diaminopimelic acid in the peptidoglycan and MK-9(H₄) as the major menaquinone. The bacterium is characterized by high proportions of the fatty acids palmitic acid, oleic acid, tuberculostearic acid and hydroxypalmitic acid. DNA–DNA hybridization analysis showed the marine bacterium to display 29.1% relatedness with *Aeromicrobium fastidiosum* DSM 10552^T and 44.4% relatedness with *Aeromicrobium erythreum* DSM 8599^T. *A. marinum* was demonstrated to be an abundant member of the pelagic bacterial community in the German Wadden Sea since it represented about 1% of the total bacterial population as revealed by dot-blot hybridization and most-probable-number counts.

The presence of Gram-positive bacteria as indigenous members of marine bacterial communities has been recognized only relatively recently. Their ubiquitous distribution in marine habitats was demonstrated by clone libraries (Fuhrman *et al.*, 1993; Rappé *et al.*, 1997; Urakawa *et al.*, 1999) or by hybridization with 16S rRNA genus-specific probes (Moran *et al.*, 1995). The clones recovered from bacterioplankton of the Sargasso Sea and the Pacific Ocean so far form a phylogenetically coherent clade (Rappé *et al.*, 1997) within the newly proposed phylum 'Actinobacteria' (Garrity & Holt, 2001) and they are distantly related to their nearest cultivated relatives (Rappé *et al.*, 1999). Recent studies even suggest that marine Gram-positive bacteria represent a large fraction of the culturable heterotrophs from sea water, marine sediments, algae and invertebrates (Jensen & Fenical, 1995; Ortigosa *et al.*, 1997; Mincer *et al.*, 2002) and most of them require sea water for

growth. This is an important trait to characterize the origin of Gram-positive bacteria from marine environments. There is an ongoing debate whether Gram-positive bacteria, especially those which were isolated from near-shore habitats, are an indigenous component of the marine bacterial community or whether they were washed in from terrestrial systems (Jensen *et al.*, 1991; Takizawa *et al.*, 1993; Bull *et al.*, 2000). The wash-in hypothesis is based on different studies which demonstrated a high degree of salt tolerance of many terrestrial streptomycetes (Okazaki & Okami, 1976) and showed that the number of actinomycetes in marine habitats decreases with increasing distance from land (Goodfellow & Haynes, 1984). Despite the evidence supporting growth of actinobacterial species in marine environments (Ortigosa *et al.*, 1997; Mincer *et al.*, 2002), only a few marine species, for example, *Rhodococcus marinonascens* (Helmke & Weyland, 1984), *Dietzia maris* (Rainey *et al.*, 1995) and *Microbacterium maritropicum* (Takeuchi & Hatano, 1998), have been characterized in detail. Thus, the ecological role of marine Gram-positive bacteria remains still unexplained. We isolated and characterized an abundant indigenous Gram-positive bacterium from surface waters of the German Wadden Sea. This organism is affiliated with the genus *Aeromicrobium* and is the first marine representative of the family *Nocardioideae* within the order *Actinomycetales*.

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; MPN, most-probable-number.

The GenBank accession number of the 16S rRNA gene sequence of *Aeromicrobium marinum* T2^T is AY166703.

A comparison of the cellular fatty acids of *A. marinum* with those of related taxa is available in IJSEM Online.

Aeromicrobium erythreum DSM 8599^T and *Aeromicrobium fastidiosum* DSM 10552^T were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and were cultured as recommended by the supplier. Strain T2^T was isolated from the highest positive dilution (10⁻⁴) of a most-probable-number (MPN) series (1 ml inoculum added to 9 ml medium). Marine broth (MB; Difco Laboratories) was used as medium and was inoculated with Wadden Sea surface water sampled near the village of Neuharlingersiel 3 h before high tide. Incubation of the MPN series was carried out at 28 °C for 30 days. Total bacterial numbers in the water sample were determined by 4',6-diamidino-2-phenylindole (DAPI) staining according to Crump *et al.* (1998). Isolation and subcultivation were performed in MB or on marine agar (MA 2216; Difco Laboratories) at 28 °C. For some phenotypic characterizations, the organism was cultured in artificial sea water (ASW) modified after Levring (1946) consisting of (per litre distilled water): 23.6 g NaCl; 0.64 g KCl; 4.53 g MgCl₂.6H₂O; 5.94 g MgSO₄.7H₂O; 1.3 g CaCl₂.2H₂O; 10 mg Na₂PO₄.2H₂O; 2.1 mg NH₄NO₃. To avoid precipitation, the CaCl₂ was sterilized separately. The ASW medium contained as source of carbon 0.3 % (w/v) yeast extract, 0.6 % peptone and 0.3 % glucose (denoted as

ASW/YPG). For minimal media containing only defined carbon sources (e.g. mannitol and amino acids), 10 ml of a 10 vitamin solution (Balch *et al.*, 1979) and 1 ml of trace element solution (Widdel & Bak, 1992) were additionally supplied per litre of ASW. All incubations for the characterization experiments were carried out at 20–25 °C for 1–2 weeks. Morphological features were investigated with cells grown on MA or in MB. Colony morphology was studied using a Zeiss Axiolab microscope. Transmission electron microscopy was carried out with negatively stained cells (Cole & Popkin, 1981) using a transmission electron microscope 109 (Zeiss). Motility was checked microscopically. The ability to grow with different carbon sources was analysed in ASW supplemented with 2–5 mM of the tested carbon source. The temperature range for growth was determined in ASW/YPG between 4 and 55 °C. The pH range (4.5–10.5) was tested in a minimal medium (ASW) containing 5 mM mannitol. Growth was determined by an increase in turbidity. Gram staining, catalase and oxidase tests were performed as described by Smibert & Krieg (1994). Salinity range was determined using ASW/YPG as basis medium. Exoenzyme activities (amylase by starch hydrolysis, caseinase by casein hydrolysis, chitinase by chitin hydrolysis and cellulase by hydrolysis of cellulose) were

Table 1. Phenotypic properties that differentiate *Aeromicrobium marinum* T2^T from related taxa

+, Positive reaction or growth; –, negative reaction or no growth; ND, no data available.

Characteristic	<i>A. marinum</i> T2 ^T	<i>A. erythreum</i> DSM 8599 ^T	<i>A. fastidiosum</i> DSM 10552 ^T	<i>N. albus</i> ATCC 27980 ^T	<i>K. sandramycini</i> ATCC 39419 ^T
Cell morphology	Rods	Irregular rods, cocci	Rods, cocci	Hyphae	Hyphae
Cell size (µm)	0.7–1.3 × 0.3–0.5	0.5 × 0.5–1.2	ND	0.5–1.0 in diameter	ND
Aerial mycelium	–	–	ND	+	+
Growth on:					
Glycerine	–	+	+	+	+
Glucose	–	+	ND	+	+
Arabinose	–	+	+	+	+
Rhamnose	–	–	–	+	+
Mannose	–	–	+	+	+
Trehalose	+	+	ND	–	+
Lactose	–	–	ND	+	+
Mannitol	+	–	ND	+	+
Alanine	–	ND	+	ND	ND
Hydrolysis of:					
Starch	–	+	+	+	+
Cellulose	–	+	–	ND	ND
Casein	–	+	+	+	+
Optimum temperature for growth (°C)	25	35	25	28	20–37
DNA G + C content (mol%)	70.6	71–73	71–72	66.5–68.6	68.3
Major menaquinone MK-9(H4)	+	+	+	–	+
Isolated from	German Wadden Sea	Tropical soil	Herbage	Soil	Mexican soil
Reference(s)	This study	Miller <i>et al.</i> (1991); Schumann <i>et al.</i> (1997)	Collins & Stackebrandt (1989)	Prauser (1989); Park <i>et al.</i> (1999)	Park <i>et al.</i> (1999)

analysed on solidified ASW medium with 1.5 % (w/v) agarose and supplemented with 0.5 % (w/v) starch, 10 % (w/v) skim milk powder, 0.5 % (w/v) chitin and 1.0 % (w/v) cellulose, respectively, as described previously (Smibert & Krieg, 1994). Lipase activity was also studied on solidified ASW medium supplemented with 2 % (v/v) vegetable oil. After incubation at 20 °C, the colonies in Petri dishes were covered with a 0.5 % CuSO₄ solution and incubated for 15 min. The solution was discarded and a positive result was indicated by a bluish or green colour around the colonies. The ability to denitrify was determined in ASW/YPG as described previously (Smibert & Krieg, 1994). The peptidoglycan was analysed by TLC on cellulose plates according to the method described by Rhuland *et al.* (1955) which was modified by Hasegawa *et al.* (1983). Menaquinones were determined by HPLC as described previously (Groth *et al.*, 1996) and results were verified by electron ionization MS. Fatty acid analysis was carried out as described by Sass *et al.* (2002). Isolation of genomic DNA (Cashion *et al.*, 1977) and determination of the G + C content by HPLC (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989) followed described procedures. DNA–DNA hybridization analysis was performed by using the renaturation method of De Ley *et al.* (1970) with the modifications of Escara & Hutton (1980) and Huss *et al.* (1983). Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992). The dot-blot hybridization procedure was performed as described recently (Bruns *et al.*, 2002). In brief, a digoxigenin (DIG)-labelled probe (length 569 bp) for the detection of strain T2^T was generated from a PCR-amplified 16S rRNA gene fragment (position 341–907 according to *Escherichia coli* numbering; Brosius *et al.*, 1981) of its genomic DNA. This probe was used to hybridize 16S rRNA gene fragments amplified from the complete natural bacterial community. For calibration, different amounts of genomic DNA of strain T2^T were used for amplification, subsequently blotted and hybridized with this probe. Intensities of the hybridization signals of the natural community and of the calibration were used to estimate the proportion of DNA from strain T2^T in the community DNA. Preferential amplification of the 16S rRNA gene of strain T2^T was tested by adding different concentrations of genomic DNA of strain T2^T to 10 ng of the community DNA prior to PCR amplification and hybridization. Cross-hybridization was tested by using *A. erythreum* DSM 8599^T and *A. fastidiosum* DSM 10552^T. Hybridization signals were detected with the DIG luminescence detection kit (Roche) and Lumi-Film (Roche) according to the manufacturer's instructions. For quantification of individual spots, the image was digitized and analysed by using ZERO-DSCAN software (Scanalytics). PCR amplification of almost-complete 16S rRNA genes, purification of PCR products and subsequent sequencing analysis were performed according to Brinkhoff & Muyzer (1997). The sequence of strain T2^T was compared with similar sequences of reference organisms by BLAST search (Altschul *et al.*, 1997). Phylogenetic analysis was performed with the ARB software package (Ludwig *et al.*, 2002). For tree calculation, only sequences with more than 1300 bp

were considered. A phylogenetic tree was constructed using neighbour-joining and maximum-likelihood analyses. Alignment positions at which less than 50 % of the sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rRNA genes, which cause mistakes in tree topology.

Major phenotypic properties that differentiate strain T2^T from related taxa are summarized in Table 1. Strain T2^T is a Gram-positive, non-motile, rod-shaped bacterium. No flagella, pili or other appendages were observed (Fig. 1a, b). This morphology remained constant irrespective of cell

Fig. 1. Morphological features of *Aeromicrobium marinum* T2^T. (a, b) Transmission electron micrographs of negatively stained cells of a 10-day-old culture of strain T2^T grown in MB. (c, e) Colony morphology on MA observed by light and epifluorescence microscopy at a 50-fold magnification. (c) Haloes (marked by arrowheads) of different sizes were generated by each colony. (d) Photograph of colonies with less-contrasted haloes because they were overlaid with a DAPI solution (10 µg ml⁻¹). (e) Photograph of DAPI-stained colonies from the same microscopic field as in (d).

age. No spores were produced. Colonies were ivory and 0.5–1.0 mm in diameter, circular and convex. Diffuse haloes of different sizes were observed around the colonies with a microscope (Fig. 1c). The haloes could not be stained with DAPI, indicating that they did not consist of bacterial cells (compare Fig. 1d, e). Treatment with Coomassie blue and Alcian Blue for detection of proteins or acidic polysaccharides, respectively, led to a weak staining of the haloes by Alcian Blue, indicating that one component of the haloes may be acidic polysaccharides. We assume that exopolysaccharides are excreted to attach to surfaces, as was proposed for other bacteria (Becker, 1996; Gehrke *et al.*, 2001). Surfaces for attachment might be decaying organic material in the water column, for example, algae, since strain T2^T is able to utilize mannitol (Table 1). Mannitol is a major component of seaweed (Budavari, 1989) and it is also known to be a compound of algal exudates (Spencer, 1990). Strain T2^T is a strictly aerobic and salt-requiring bacterium showing optimal growth in ASW/YPG medium with salt concentrations in the range 6.3–107‰ and a maximum at 53.5‰. The growth rate at 0.8‰ was significantly lower and no growth was observed when no salts were added. Besides the results shown in Table 1, strain T2^T showed growth with cellobiose, succinate, fumarate, pyruvate, crotonic acid, 2-oxoglutarate, arginine and glutamic acid as sole sources of carbon and energy. No growth was observed with fructose, xylose, sucrose, maltose, citrate, ethanol, propanol, butanol, pimelic acid, inositol, benzoic acid, nicotinic acid, salicylic acid, asparagine, aspartic acid, cysteine, glutamine, glycine and histidine. In contrast to

related organisms, strain T2^T did not hydrolyse starch and casein (Table 1).

The major cellular fatty acids of strain T2^T were similar to those of its nearest relatives, *A. erythreum* DSM 8599^T and *A. fastidiosum* DSM 10552^T: hexadecanoic acid (C_{16:0}, palmitic acid), *cis*-9-octadecenoic acid (C_{18:1} ω9c, oleic acid), 10-methyl octadecanoic acid (10-Me C_{18:0}, tuberculostearic acid) and 2-hydroxy hexadecanoic acid (2-OH C_{16:0}, hydroxypalmitic acid). The fatty acid profiles of the *Aeromicrobium* species can be clearly distinguished from those of their close relatives *Nocardioideus albus* ATCC 27980^T and *Kribbella sandramycini* ATCC 39419^T, which were characterized by other major fatty acids (compare data in supplementary table in IJSEM Online; <http://ijs.sgmjournals.org>). The cellular fatty acid profiles clearly distinguish the three *Aeromicrobium* species from their relatives. It is noteworthy that the major fatty acid tuberculostearic acid (10-methyl octadecanoic acid) was not found to the same extent in any other closely related species within the suborder *Propionibacterineae* (Schumann *et al.*, 1997; Park *et al.*, 1999). However, it was found in other genera within the order *Actinomycetales*, i.e. *Mycobacterium*, *Microbispora*, *Micromonospora*, *Arthrobacter*, *Nocardia*, certain *Streptomyces* spp. (Lechevalier, 1977) and *Dietzia* (Rainey *et al.*, 1995).

Comparison of the almost-complete 16S rRNA gene sequence of strain T2^T with sequences from GenBank demonstrated that this strain is the first described marine bacterium affiliated to the family *Nocardioideaceae* within the order *Actinomycetales*. The phylogenetic tree shown in Fig. 2

Fig. 2. Unrooted maximum-likelihood tree showing the relationship of *Aeromicrobium marinum* T2^T to members of the order *Actinomycetales*. Bar, 10% sequence dissimilarity.

demonstrates that strain T2^T forms a cluster with the two previously described *Aeromicrobium* species. Similarities between the 16S rRNA gene sequence of strain T2^T and those of *A. fastidiosum* DSM 10552^T and *A. erythreum* DSM 8599^T were 97.7 and 97.0 %, respectively. Other members of the *Actinomycetales* can be readily distinguished from this branch (Fig. 2). The percentage of DNA–DNA relatedness was 44.4 % for strain T2^T and *A. erythreum* DSM 8599^T, and 29.1 % for strain T2^T and *A. fastidiosum* DSM 10552^T. The reference strains *A. erythreum* DSM 8599^T and *A. fastidiosum* DSM 10552^T exhibited a DNA–DNA relatedness of 30.7 %. According to Wayne *et al.* (1987), the phylogenetic definition of a species generally includes strains with more than 70 % DNA–DNA relatedness. Thus, strain T2^T is clearly distinguishable from the existing *Aeromicrobium* species.

Two isolates with identical 16S rRNA gene sequences to that of strain T2^T were recovered in parallel to T2^T from high dilutions (10^{-4}) of a MPN series inoculated with a water sample from the German Wadden Sea. Assuming that these bacteria were identical or highly related and belong, together with T2^T, to the same species this corresponds to a cell number of $1 \times 10^4 \text{ ml}^{-1}$ of the respective species in the environmental sample. On the basis of the total bacterial cell number of $1 \times 10^6 \text{ ml}^{-1}$ in the original water sample, the MPN results indicate an abundance of 1 % of this species within the bacterial community. To confirm this result, we determined the amount of PCR-generated 16S rRNA gene fragments of strain T2^T amplified from the complete natural bacterial community, by dot-blot analysis (Bruns *et al.*, 2002). Tests with the addition of ~100- to 500-fold excess of genomic DNA of the natural community prior to PCR and hybridization analysis showed no interference with the detection of strain T2^T DNA from a pure culture. The intensities of the hybridization signals for strain T2^T alone or in combination with the natural community remained similar (compare first and second lane in Fig. 3). Some cross-hybridization was observed with DNA from *A. erythreum* DSM 8599^T; however, the intensity of the hybridization signal was only 10 % of that obtained with the same concentration of DNA of strain T2^T (Fig. 3). DNA of *A. fastidiosum* DSM 10552^T gave only very faint hybridization signals (data not shown). This is probably due to nine non-homologous bases within the 16S rRNA gene sequence of *A. fastidiosum* DSM 10552^T versus only six non-homologous bases within the sequence of *A. erythreum* DSM 8599^T when aligned with the probe sequence. The weak signals obtained with *A. erythreum* DSM 8599^T and *A. fastidiosum* DSM 10552^T also indicate the specificity of the probe and the hybridization conditions developed for the detection of strain T2^T. Based on the results of our dot-blot procedure, genomic DNA of strain T2^T and possibly of very closely related strains represents ≤ 1 % of the total genomic DNA of the natural community (Fig. 3). These results are consistent with those of the MPN counts and indicate a high abundance (~1 %) of these bacteria in the natural pelagic community. High amounts of Gram-positive

bacteria may result from wash-in events of terrestrial bacteria, as has been discussed previously (Goodfellow & Haynes, 1984). However, the fact that strain T2^T is an obligately salt-requiring bacterium and shows its optimal growth at sea water salinities gives striking evidence for its indigenous abundance in a marine habitat, as demonstrated

Fig. 3. Quantification of 16S rRNA gene fragments of *Aeromicrobium marinum* T2^T in the natural pelagic community of the German Wadden Sea by dot-blot hybridization. (a) Digitized image of an exposed Lumi-Film. (b) The different amounts of genomic template DNA used for PCR amplification are given in picograms. Different concentrations of template DNA of strain T2^T served as standards. As a control for a preferential amplification, DNA of strain T2^T was mixed with a constant concentration (10 ng) of template DNA from the natural community.

for other marine Gram-positive bacteria (Jensen *et al.*, 1991; Jensen & Fenical, 1995).

Description of *Aeromicrobium marinum* sp. nov.

Aeromicrobium marinum (ma.ri'num. L. neut. adj. *marinum* of the sea, marine).

Aerobic. Cells are Gram-positive, non-motile rods that are 0.7–1.3 µm long and 0.3–0.5 µm wide. Ivory-coloured colonies are 0.5–1.0 mm in diameter and are characterized by a diffuse halo. Grows between 4 and 35 °C, with optimum growth at 25 °C. Growth occurs between pH 5.5 and 9.5, with optimum growth at pH 7.0–8.5. The optimal salinity for growth is 53.5‰; growth is possible between 0.8 and 107‰. Catalase-positive and oxidase-negative. Does not reduce nitrate. Grows with the carbon sources trehalose, cellobiose, crotonic acid, 2-oxoglutarate, succinate, fumarate, mannitol, pyruvate, arginine and glutamic acid. Unable to hydrolyse starch, cellulose, lipids, chitin and casein. Contains LL-diaminopimelic acid as the diamino acid in the cell-wall peptidoglycan. The major menaquinone is MK-9(H₄). The cellular fatty acids are predominantly hexadecanoic acid (palmitic acid), *cis*-9-octadecenoic acid (oleic acid), 10-methyl octadecanoic acid (tuberculostearic acid) and 2-hydroxy hexadecanoic acid (hydroxypalmitic acid).

The type strain is T2^T (= DSM 15272^T = LMG 21768^T). The G+C content of its DNA is 70.6 mol%. Isolated from surface waters of the German Wadden Sea.

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